# Age-Induced Changes in Mu Opioid Receptor Signaling in the Midbrain Periaqueductal Gray of Male and Female Rats

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**Highlights:**

- Aged males and females (adult and aged) exhibit reduced vlPAG μ-opioid receptor binding potential compared to adult males.
- Aged males and females (adult and aged) exhibit reduced opioid-induced vlPAG G-protein activation compared to adult males.
- Aged males and females (adult and aged) exhibit reduced vlPAG MOR mediated cAMP inhibition compared to adult males.
- Aged rats (males and females) exhibit increased vlPAG mRNA expression of Regulator of G-Protein Signaling (RGS) proteins RGS4 and RGS9-2 compared to adult rats (males and females), which may explain the reduced receptor signaling observed in aged animals.
- These coordinate decreases in opioid receptor signaling may explain the previously reported reduced potency of opioids to produce pain relief in females and aged rats.

**Abstract: The analgesic effects of opioids are attenuated in aged rats.** Opioids such as morphine have decreased analgesic potency (but not efficacy) in aged rodents compared to adults; however, the neural mechanisms underlying this attenuated response are not yet known. The present study investigated the impact of advanced age and biological sex on opioid signaling in the ventrolateral periaqueductal gray (vlPAG) in the presence of chronic inflammatory pain. Assays measuring mu-opioid receptor (MOR) radioligand binding, GTPγS binding, receptor phosphorylation, cAMP inhibition, and regulator of G-protein signaling (RGS) protein expression were performed on vlPAG tissue from adult (2-3mos) and aged (16-18mos) male and female rats. Persistent inflammatory pain was induced by intraplantar injection of Complete Freund's Adjuvant (CFA). Adult males exhibited the highest MOR binding potential and the highest G-protein activation (activation efficiency ratio) in comparison to aged males and females (adult and aged). No impact of advanced age or sex on MOR phosphorylation state was observed. DAMGO-induced cAMP inhibition was highest in the vlPAG of adult males compared to aged males and females (adult and aged). vlPAG levels of RGS4 and RGS9-2, critical for terminating G-protein signaling, were assessed using RNAscope. Adult rats (both males and females) exhibited lower levels of vlPAG RGS4 and RGS9-2 mRNA expression compared to aged males and females. The observed age-related reductions in vlPAG MOR binding potential, G-protein activation efficiency, and cAMP inhibition, along with the observed age-related increases in RGS4 and RGS9-2 vlPAG expression, provide potential mechanisms whereby the potency of opioids is decreased in the aged population. These results have significant implications for pain management in this population.

**Keywords:** chronic pain; opioid signaling; pharmacology; advanced age; sex differences; mu-opioid receptor

24 **1 Introduction**

25 Chronic pain is a debilitating condition that contributes to mental health disorders, disability, and
26 increased risk of premature death (Domenichiello and Ramsden, 2019; Patel et al., 2013). More than 50%
27 of individuals aged 65 and over suffer from chronic pain, and as the United States population rapidly ages,
28 the development of effective strategies for chronic pain management for this demographic is imperative
29 (Dahlhamer, 2018). Opioids, including morphine and fentanyl, are commonly prescribed analgesics for the
30 management of chronic pain (Manchikanti et al., 2010; Sullivan et al., 2010), but effective dosing strategies
31 for the elderly remain ill-defined, due in large part to a dearth of knowledge regarding the impact of
32 advanced age on opioid signaling and analgesia.

33 Clinical studies examining pain management in the elderly are challenging; co-morbid conditions such as
34 diabetes and high blood pressure, and participant use of concomitant medications affect patient
35 outcomes, contributing to difficulties in the interpretation of results (Naples et al., 2016; Prostran et al.,
36 2016). Additionally, there exist age- and sex-related individual differences in the likelihood of reporting
37 pain in a clinical setting which may lead to misrepresentations of analgesic efficacy (Dampier et al., 2013;
38 Reddy et al., 2012). Particularly, aged populations are known to underreport pain due to fears of
39 institutionalization and concerns with addiction and overdose (Reddy et al., 2012).

40 Using a preclinical model of persistent inflammatory pain (intraplantar Complete Freund's Adjuvant;
41 (CFA)), we have previously reported a significant impact of age and sex on morphine potency. Specifically,
42 we showed that aged rats (18mos) exhibit a decreased morphine potency compared to adults (2mos), with
43 aged males requiring greater than 2x the concentration of morphine than their adult counterparts to
44 produce equivalent analgesia (Fullerton et al., 2021). Similar results have been reported in prior preclinical
45 studies, suggesting that aged rodents require higher doses of opioids to produce antinociception (Kavaliers

46 et al., 1983; Kramer and Bodnar, 1986; Webster et al., 1976). The mechanisms underlying the attenuation
47 of opioid potency in the aged population are currently unknown.

48 Morphine-induced analgesia is mediated primarily via binding to μ-opioid receptors (MOR), prototypical
49 G-protein coupled receptors (GPCRs) located predominantly on neuronal cell membranes (Martin, 1963;
50 Wolozin and Pasternak, 1981). Following agonist binding, coupled G-proteins undergo a conformational
51 change in which the guanosine diphosphate (GDP) bound to the inactive alpha subunit is replaced by
52 guanosine triphosphate (GTP), activating the subunit and promoting opioid signaling through interaction
53 with downstream effectors (Senese et al., 2020). MORs are coupled to a family of G-proteins called Gi/o
54 that signal via inhibition of adenylyl cyclase and subsequently decrease cyclic adenosine monophosphate
55 (cAMP) (Koehl et al., 2018; Bouchet et al., 2021). This G-protein signaling is downregulated or terminated
56 by **R**egulator of **G**-Protein **S**ignaling (RGS) proteins, which act as GTPase activating proteins (GAPS),
57 hydrolyzing the active GTP back into GDP and terminating downstream signaling (Gerber et al., 2016). RGS
58 proteins, particularly RGS4 and RGS9-2, have been previously implicated in opioid-mediated G-protein
59 signaling, and have been shown to attenuate analgesia (Garnier et al., 2003; Psifogeorgou et al., 2007;
60 Avrampou et al., 2019; Senese et al., 2020). A primary target of MOR downstream signaling is the effector
61 adenylate cyclase (AC). AC enzymatically converts adenosine triphosphate (ATP) to the excitatory second
62 messenger cyclic adenosine monophosphate (cAMP). MOR binding inhibits AC, thereby reducing cAMP
63 expression, facilitating neuronal hyperpolarization, and promoting analgesia (Santhappan et al., 2015). Like
64 other G-protein coupled receptors, MOR signaling is subject to desensitization via the cellular mechanism
65 of receptor phosphorylation, which limits agonist binding and recruits arrestin proteins, and thus
66 attenuates opioid signaling. (Zhang et al., 1996; Yu et al., 1997; Groer et al., 2011).

67 The present studies test the hypothesis that the observed age-induced reduction in morphine potency in
68 the presence of chronic pain is mediated by changes in MOR signaling within the midbrain periaqueductal

69 gray, a CNS region critical for the opioid modulation of pain. (Basbaum et al., 1976; Behbehani and Fields,
70 1979; Morgan et al., 2006, 1992). The ventrolateral PAG (vlPAG) contains a large population of MOR+
71 neurons, and direct administration of MOR agonists into the PAG produces potent analgesia (Satoh et al.,
72 1983; Jensen and Yaksh, 1986; Bodnar et al., 1988; Loyd et al., 2008) In addition, intra-PAG administration
73 of MOR antagonists or lesions of PAG MOR significantly attenuate the analgesic effect of systemic
74 morphine, suggesting a critical role for PAG MOR in mediating morphine action (Ma and Han, 1991; Y.
75 Zhang et al., 1998; Loyd et al., 2008).

76 We have previously shown that aged rats exhibit reduced vlPAG MOR protein expression and reduced
77 MOR agonist binding in the vlPAG compared to adult rats (Fullerton et al., 2021), suggesting that
78 diminished levels of functioning MOR in the vlPAG contribute to the attenuated opioid potency seen in the
79 aged. The present studies build on these previous findings to assess age and sex differences in MOR
80 availability, ligand affinity, phosphorylation, G-protein activation, cAMP inhibition, and expression of RGS
81 proteins. We report that aged rats, and more specifically males, exhibit reduced MOR binding potential,
82 reduced G-protein activation, reduced cAMP inhibition, and increased RGS expression compared to adults,
83 with no change in MOR phosphorylation. Similar changes were observed in females regardless of age.
84 These findings identify several mechanisms whereby opioid signaling is attenuated in the aged rat, and
85 provide a framework for the development of novel pharmacological therapies to improve pain
86 management in the elderly.

87 **2 Materials and methods**

88 **2.1 Experimental subjects**

89 Adult (2-3mos) and aged (16-18mos) male and regularly cycling female Sprague–Dawley rats were used
90 in these experiments (Charles River Laboratories, Boston, MA). Rats were co-housed in same-sex pairs
91 on a 12:12 hour light/dark cycle (lights on at 08:00 am). Access to food and water was ad libitum

92 throughout the experiment, except during testing. All studies were approved by the Institutional Animal
93 Care and Use Committee at Georgia State University and performed in compliance with Ethical Issues of
94 the International Association for the Study of Pain and National Institutes of Health. All efforts were
95 made to reduce the number of rats used in these experiments and to minimize pain and suffering. All
96 assays were performed on separate cohorts of rats (n=8/sex/age; N=32), with the exception of the
97 radioligand binding and GTPγS assays, which were run simultaneously on PAG tissue from a single
98 cohort.

99 **2.2 Vaginal cytology**

100 Beginning ten days prior to testing, vaginal lavages were performed daily on adult and aged female rats
101 to confirm that all rats were cycling regularly and to keep daily records of the stage of estrous. Proestrus
102 was identified as a predominance of nucleated epithelial cells, and estrus was identified as a
103 predominance of cornified epithelial cells. Diestrus 1 was differentiated from diestrus 2 by the presence
104 of leukocytes. Rats that appeared between phases were noted as being in the more advanced stage
105 (Loyd et al., 2007).

106 **2.3 CFA-induced chronic pain treatment**

107 72 hours prior to experimentation, persistent inflammatory pain was induced by injection of complete
108 Freund's adjuvant (CFA; Mycobacterium tuberculosis; Sigma; 200µl), suspended in an oil/saline (1:1)
109 emulsion, into the plantar surface of the right hind paw. Edema was present within 24 hours of
110 injection, indicated by a >100% change in paw diameter, determined using calibrated calipers applied
111 midpoint across the plantar surface compared to handled paw.

112 **2.4 Membrane preparation for radioligand binding and GTPγS assays**

113 vlPAG membrane protein lysates were prepared from adult and aged, male and female, handled and
114 CFA treated rats to be used for radioligand binding and GTPγS assays. 72 hours post-CFA injection or
115 handling, rats were restrained using DecapiCones and decapitated. Brains were removed rapidly, flash-
116 frozen in 2-methyl butane on dry ice, and stored at −80°C. Ventrolateral PAG tissue from caudal PAG
117 (Bregma -7.5 through -8.5) was dissected from each brain using a straight edge razor at -20°C. On the
118 day of the assay, PAG sections were placed in ice-cold assay buffer (50 mM Tris-HCl, pH 7.4). Tissue was
119 homogenized with a glass dounce and centrifuged at 20,000g at 4°C for 30 min. The supernatant was
120 discarded and the pellet resuspended in assay buffer (Li et al., 2018; Shaqura et al., 2016b, 2016a;
121 Zollner et al., 2003). Membrane protein concentration was calculated using the Bradford Assay, and
122 lysates of vlPAG membrane protein from adult and aged, male and female, naïve and CFA treated rats
123 (n=4; N=32) were used immediately for radioligand binding and GTPγS assays.

124 **2.5 Saturation radioligand binding assay**

125 Saturation binding experiments were performed on vlPAG membranes using [$^3$H]DAMGO (specific
126 activity 50 Ci/mmol, American Radiolabeled Chemicals, Missouri). Briefly, 100 µg of membrane protein
127 was incubated with various concentrations of [$^3$H]DAMGO (0.5 to 5nM), in a total volume of 1ml of
128 binding buffer (50mM Tris-HCl pH 7.4). Nonspecific binding was defined as radioactivity remaining
129 bound in the presence of 10 µM unlabeled naloxone. At the end of the incubation period (1h at RT)
130 bound and free ligands were separated by rapid filtration over Whatman brand Grade GF/C glass filters
131 (Sigma-Aldrich) using a sampling vacuum manifold (MilliporeSigma). Filters were washed four times with
132 5ml of cold $dH_20$. Bound radioactivity was determined by liquid scintillation spectrophotometry after
133 overnight extraction of the filters in 3ml of scintillation fluid. Specific activity was calculated by
134 subtracting the non-specific binding mean value at each concentration of [$^3$H]DAMGO. All experiments
135 were performed in triplicate. $B_{max}$ and $K_d$ values were determined by nonlinear regression analysis of
136 concentration-effect curve in GraphPad Prism 9.1.

137 **2.6 Agonist-stimulated [$^{35}$S]GTPγS binding**

138 DAMGO-stimulated [$^{35}$S]GTPγS binding to PAG membrane protein was assessed by incubating
139 membrane protein (100ug) in the presence or absence of [$^{35}$S]GTPγS (0.1nM) (specific activity 1250
140 Ci/mmol, American Radiolabeled Chemicals, Missouri) and various concentrations of DAMGO (2 to
141 30,000 nM) in assay buffer (20mM Tris, 10mM $MgCl_2$, 100mM NaCl, 0.2mM EGTA, pH 7.4) for 30min at
142 30°C. Stimulated [$^{35}$S]GTPγS binding was compared to unstimulated binding at each measurement point
143 and presented as percent basal binding. At the end of the incubation period, bound and free ligands
144 were separated by rapid filtration over Whatman brand Grade GF/B glass filters (Sigma-Aldrich) using a
145 sampling vacuum manifold (MilliporeSigma). Filters were washed four times with 5ml of cold buffer (50
146 mM Tris-HCl, pH 7.4). Bound radioactivity was determined by liquid scintillation spectrophotometry
147 after overnight extraction of the filters in 3ml of scintillation fluid. All experiments were performed in
148 duplicate. Efficacy (Emax) is defined as the maximum percent stimulation by an agonist; potency ($EC_{50}$) is
149 defined as the concentration of DAMGO required for half the maximal response. Emax and $EC_{50}$ values
150 were determined by nonlinear regression analysis of concentration-effect curves using Graph Pad Prism
151 9.1.

152 **2.7 Phosphorylated MOR analysis**

153 To assess the impact of advanced age and sex on MOR phosphorylation state, levels of MOR
154 phosphorylation were analyzed by Western blot. The lysis buffers used and general methods are the
155 same as reported in (Lei et al., 2017). Briefly, rat PAG samples were homogenized in ~500 µL of lysis
156 buffer using a glass dounce and rotated overnight at 4°C. The samples were then spun down at 13,000g
157 for 10 min at 4°C. The resulting lysates were quantified by DC Protein Assay Kit (Bio-Rad, #500-0111)
158 according to the manufacturer's instructions. 50-75 µg of soluble protein per sample (same for all
159 samples in a set) was run on 10% Bis-Tris Bolt PAGE gels (Fisher Scientific, #NW00100BOX), and wet-
160 transferred to nitrocellulose membrane (Protran 0.2 µm NC, #45-004-001 from Fisher Scientific) at 30V

161 at 4°C for 2-3 hours. Membranes were blocked using 5% nonfat dry milk in TBS, then blotted for primary
162 antibody target in 5% BSA in TBST overnight rocking at 4°C. Primary antibodies used were: pMOR
163 (1:1000, Bioss #bs-3724R), tMOR (1 µg/mL, R&D #MAB6866), and GAPDH (1:1000, Invitrogen #MA5-
164 15738). All 3 targets were always probed for on the same blot, using low pH stripping buffer between
165 each set. Secondary antibodies were used at 1:5000 each in 5% nonfat dry milk in TBST, rocking for ~1 hr
166 at room temperature. Secondary antibodies used were Goat-α-Mouse-IRDye800CW (Fisher Scientific
167 #NC9401841) and Goat-α-Mouse-IRDye680LT (Fisher Scientific #NC0046410). Target signal was acquired
168 using an Azure Sapphire imager using the near-infrared channels (658 and 784 nm). Band density was
169 quantified, and background subtracted using the onboard AzureSpot analysis software. Samples were
170 run with at least two representatives of each experimental group on the same gel. Adult and aged, male
171 and female, naïve and CFA treated rats (n=4; N=32) were used for these experiments. pMOR signal was
172 normalized to the same sample tMOR or GAPDH as indicated, and all samples were normalized to the
173 Adult Male average on each gel before combining data from different gels.
174

175 **2.8 Agonist-dependent cAMP inhibition**

176 DAMGO-dependent cAMP inhibition was analyzed using a cAMP assay. vlPAG membrane protein lysates
177 were prepared from adult and aged, male and female, handled and CFA treated rats. 72 hours post-CFA
178 injection or handling, rats were restrained using DecapiCones and decapitated. Brains were removed
179 rapidly, flash-frozen in 2-methyl butane on dry ice, and stored at −80°C. Ventrolateral PAG tissue from
180 caudal PAG (Bregma -7.5 through -8.5) was dissected from each brain using a straight edge razor at -
181 20°C. Briefly, vlPAG tissues were homogenized in ice-cold lysis buffer containing 0.25-M sucrose, 50-mM
182 Tris–HCl pH 7.5, 5-mM EGTA, 5-mM EDTA, 1-mM phenylmethylsulfonyl fluoride, 0.1-mM dithiothreitol,
183 and 10 µg/ml leupeptin. The homogenized tissues were then centrifuged at 1000×*g* for 5 min (4 °C) and
184 the supernatant was centrifuged at 35000 ×*g* for 10 min (Viganò et al., 2003). vlPAG membrane protein

185 samples (100ug) from adult and aged, male and female, naïve and CFA treated rats (n=4; N=32) were
186 incubated in 1uM forskolin (FSK) and in the presence or absence of 10uM DAMGO to stimulate
187 adenylate cyclase activity. cAMP levels in vlPAG tissue were determined using the LANCE *Ultra* cAMP
188 Detection Kit (Perkin Elmer) a time-resolved fluorescence resonance energy transfer (TR-FRET) cAMP
189 immunoassay. Data are plotted as % change in TR-FRET signal comparing FSK baseline measurements to
190 measurements following DAMGO-stimulated cAMP inhibition.

191 **2.9 Single-molecule fluorescence in situ hybridization**

192 Single-molecule fluorescent in situ hybridization (RNAscope) assays were used to determine mRNA
193 expression of OPRM1, RGS9-2, and RGS4 in the vlPAG. Rats were restrained using DecapiCones and
194 decapitated. Brains were removed rapidly, flash frozen in 2-methyl butane on dry ice, and stored RNase
195 free at −80 °C. Frozen tissue was sectioned in a 1:6 series of 20 µm coronal sections at –20 °C with a
196 Leica CM3050S cryostat. Sections were immediately mounted onto Superfrost slides (20 °C) and stored
197 at −80 °C until the time of the assay. vlPAG sections from adult and aged, male and female, naïve and
198 morphine treated rats were used (n=4; N=32). For morphine dosing paradigm see (Fullerton et al.,
199 2021). Tissue was processed for single-molecule fluorescence in situ hybridization (smFISH) according to
200 the RNAscope Multiplex Kit protocol (Advanced Cell Diagnostics) using probes for OPRM1, RGS4, and
201 RGS9-2. To facilitate cellular mRNA quantification within the vlPAG, sections were counterstained with
202 DAPI. mRNA puncta were visualized as fluorescent signal. Fluorescent images were captured on Zeiss
203 LSM 700 Confocal Microscope at 40x, and mRNA expression (target puncta/DAPI) was calculated using
204 Imaris software. To determine RGS4 and RGS9-2 expression levels in MOR+ neurons, quantification was
205 restricted to puncta located within 10µm of DAPI that co-expressed OPRM1 mRNA. mRNA expression
206 values were determined for the left and right ventrolateral subdivisions of each PAG image from two
207 representative levels of the mid-caudal PAG (Bregma −7.74 and −8.00). As there was no significant effect
208 of rostrocaudal level in the analyses, data were collapsed and presented as vlPAG and were averaged for

209 each rat. RGS4 and RGS9-2 mRNA values are expressed as the mean ± standard error of the mean (SEM).
210 All images were collected and analyzed by an experimenter blinded to the experimental condition.

211 **2.10 Statistical analysis and data presentation**

212 All values are reported as mean ± SEM. Data were assessed for normality and homogeneity of variance
213 using Shapiro-Wilk and Bartlett's tests. Significant main effects of sex, age, and treatment were assessed
214 using ANOVA; $p < 0.05$ was considered statistically significant. Tukey's post-hoc tests were conducted to
215 determine significant mean differences between groups that were a priori specified. Data are expressed
216 either as fmol/protein or nM DAMGO for radioligand binding assay, or Emax or EC50 for GTPγS assay.

217 **3 Results**

218 **3.1 Advanced age and sex impact vlPAG MOR binding properties**

219 To assess the impact of advanced age, biological sex, and pain on vlPAG MOR signaling, we first used
220 radioligand saturation binding assays to determine MOR binding parameters. The saturation binding
221 curves generated with [$^3$H]DAMGO are shown in Fig. 1A. There was no significant impact of CFA
222 treatment on any measures, so CFA and handled groups were combined.

223 Kd values, indicative of receptor affinity, were determined using the concentration-effect curves
224 generated from each sample. Analysis of Kd values indicated no significant impact of age [$F_{(1,26)} = 0.770$,
225 $p=0.388$] or sex [$F_{(1,26)} = 1.241$, $p=0.275$], or a significant interaction [$F_{(1,26)} = 1.278$, $p=0.269$] (Fig. 1B).

226 Analyses of $B_{max}$ values, indicative of receptor availability, showed no significant impact of age [$F_{(1,28)}$ =
227 2.661, $p=0.114$] or sex [$F_{(1,28)} = 0.002$, $p=0.967$], but a significant interaction [$F_{(1,28)} = 4.995$, $p=0.034$]. Post
228 hoc analysis showed a significant difference in vlPAG MOR availability between adult males and aged
229 males, as evidenced by reduced $B_{max}$ values in the aged males compared to their adult counterparts ($p$ =
230 0.025)(Fig. 1C). $B_{max}$ values for aged males were markedly reduced compared to adult males, while adult

231 females and aged females exhibited smaller reductions in $B_{max}$ compared to their adult male
232 counterparts (%Δ -23.6 and -17.1, respectively. Fig. 1C).

233 Analyses of binding potential (BP) values, a measure that takes into consideration the density of
234 available receptors and the affinity of the receptor for its agonist, showed a significant impact of sex,
235 with males exhibiting greater binding potential than females [$F_{(1,28)} = 4.631$, $p=0.040$] and a significant
236 interaction between age and sex [$F_{(1,28)} = 9.110$, $p=0.005$]. No significant impact of age [$F_{(1,28)} = 3.312$,
237 $p=0.079$] was observed. Post hoc analyses showed a significant difference in vlPAG MOR binding
238 potential between adult males and adult females ($p = 0.006$), and adult males and aged males ($p =$
239 $0.010$), indicating that adult males exhibit greater vlPAG MOR binding potential than their aged male
240 counterparts and their adult female counterparts (Fig. 1D). Adult females, aged males, and aged females
241 all exhibited marked reductions in MOR binding potential compared to their adult male counterparts
242 (%Δ -52, -48.7, and -40, respectively; Fig. 1D).

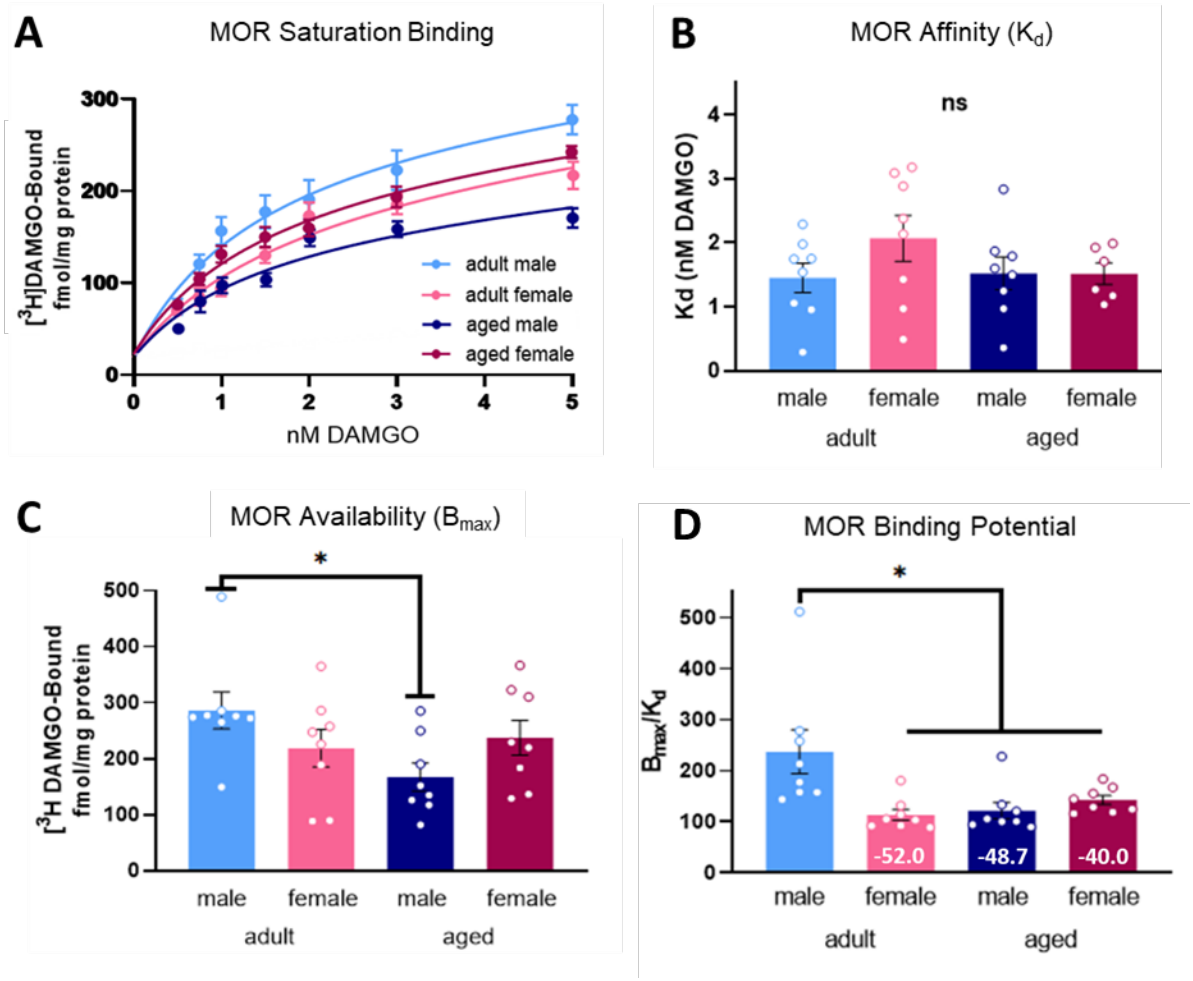



**Fig. 1.** Saturation binding curve of bound [3H]DAMGO (A). No significant effect of age or sex was observed in MOR affinity ($K_d$) (B). MOR availability ($B_{max}$) was significantly lower in aged males compared to adult males (C). Adult females, aged males, and aged females exhibited reduced MOR binding potential ($B_{max}/K_d$) compared to adult males, indicating attenuated agonist interaction at the receptor level (D). ns, not significant. *Significant difference between adult and aged males, or adult males and adult females; $p<0.05$ calculated by Tukey's post hoc test. Graphs indicate mean ± SEM. Values indicate % change from adult male.

### 3.2 Advanced age and sex impact opioid-induced G-protein activation in the vlPAG

We next conducted GTPγS binding assays to determine if advanced age or biological sex impacted MOR mediated G-protein activation. Preliminary studies revealed a significant main effect of chronic pain [$F_{(1, 23)} = 21.16$, $p = 0.0001$]. To improve the translatability of these results to the target population of aged patients suffering from chronic pain, GTPγS experiments were performed exclusively on CFA-treated

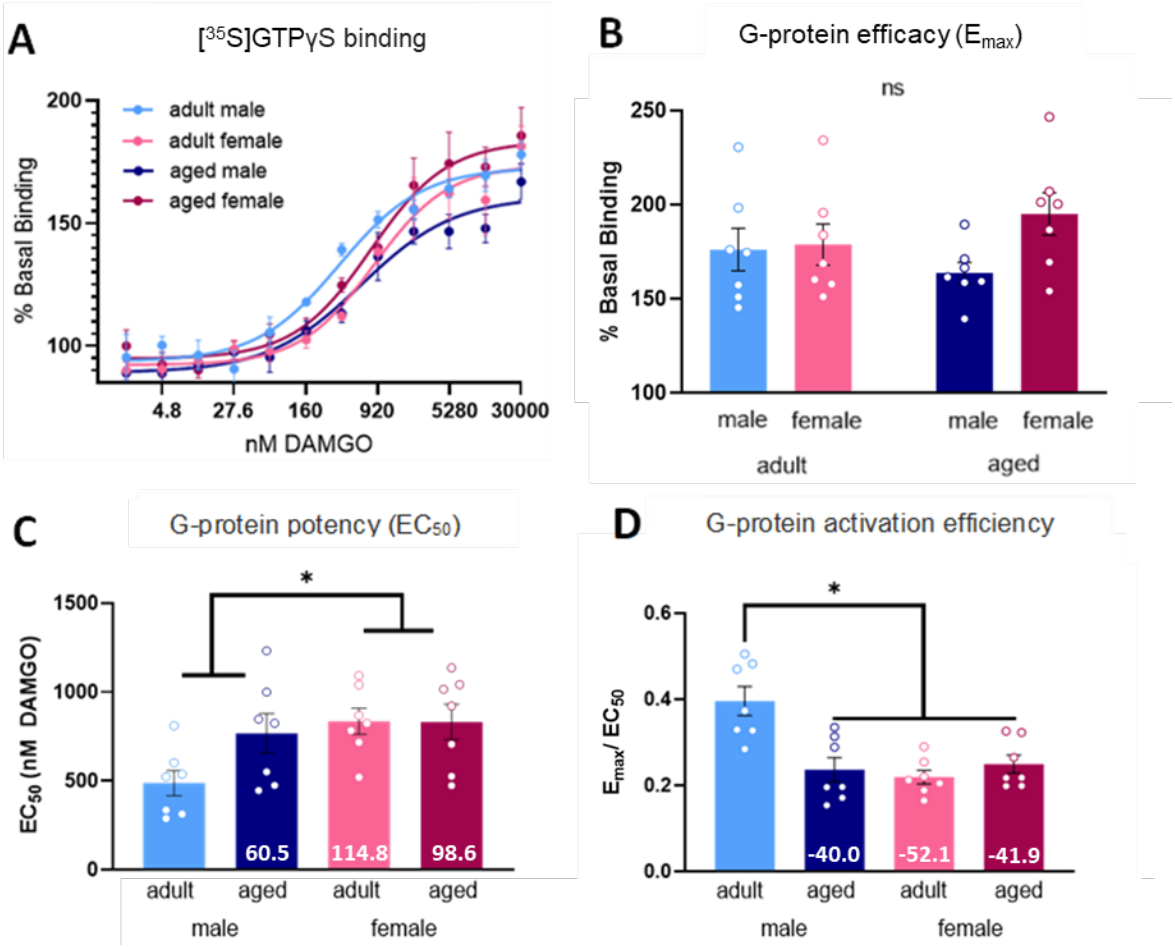
256 rats. Concentration curves generated from [$^{35}$S]GTPγS assays are shown in Fig. 2A. Analyses of $E_{max}$
257 values, a measure of G-protein availability combined with ligand efficacy, indicated no significant impact
258 of age [$F_{(1,24)}$ = 0.035, p = 0.8532] or sex [$F_{(1,25)}$ = 2.852, p = 0.1042], or a significant interaction [$F_{(1,24)}$ =
259 2.023, p = 0.1678] (Fig. 2B).

260 Analyses of $EC_{50}$ values, a measure of the concentration of DAMGO required for half-maximal G-protein
261 binding, indicated a significant impact of sex, with males exhibiting lower effective concentration values
262 than females, reflecting a higher potency of activation [$F_{(1,24)}$ = 5.177, p = 0.0321]. There was no
263 significant impact of age [$F_{(1,24)}$ = 2.339, p = 0.1393], and no significant interaction [$F_{(1,24)}$ = 2.473, p =
264 0.1289] (Fig. 2C). Compared to their adult male counterparts, aged males, adult females, and aged
265 females all exhibited marked increases in G-protein $EC_{50}$ as evidenced by their percent change values
266 (%Δ 60.5, 114.8, and 98.6, respectively), reflecting a lower potency of activation (Fig. 2C). Together,
267 these results suggest that opioid-induced G-protein signaling is impaired in the PAG of the aged rat and
268 the female rat.

269 Next, we calculated the coefficient ratio of analysis of $E_{max}$ / $EC_{50}$, which is indicative of overall G-protein
270 activation efficiency. A significant impact of age was noted, with adults exhibiting higher vlPAG G-
271 protein activation efficiency than aged rats [$F_{(1,24)}$ = 6.491, p = 0.0177]. There was also a significant
272 impact of sex, with males exhibiting higher vlPAG G-protein activation efficiency compared to females
273 [$F_{(1,24)}$ =10.39, p = 0.0036]. A significant interaction was also observed [$F_{(1,24)}$ = 13.93, p = 0.0010]. Post
274 hoc analyses showed a significant difference in vlPAG G-protein activation efficiency between adult
275 males and aged males (p = 0.0009), with adult males exhibiting greater vlPAG G-protein activation
276 efficiency than aged males. A significant difference in vlPAG G-protein activation efficiency between
277 adult males and adult females (p= 0.0003) was also observed, with adult males exhibiting greater vlPAG
278 G-protein activation efficiency than adult females (Fig. 2D). Aged males, adult females, and aged females

279 all exhibited marked reductions in G-protein activation efficiency compared to their adult male

280 counterparts (%Δ -40.0, -52.1, and -41.9, respectively; Fig. 2D).




281

282 **Fig 2.** [$^{35}$S]GTPγS binding curve of agonist-stimulated GTPγS binding (A). No significant effect of age or
283 sex was observed in $E_{max}$ (B). Aged males, adult females, and aged females exhibited increased $EC_{50}$
284 values compared to adult males, indicating that adult males have the greatest potency (C). Aged males,
285 adult females, and aged females exhibited reduced G-protein activation efficiency ($E_{max}/EC_{50}$) compared
286 to adult males, indicating attenuated G-protein signaling (D). ns, not significant. *Significant difference
287 between adult and aged males, or adult males and adult females; $p<0.05$ calculated by Tukey's post hoc
288 test. Graphs indicate mean ± SEM. Values indicate % change from adult male.

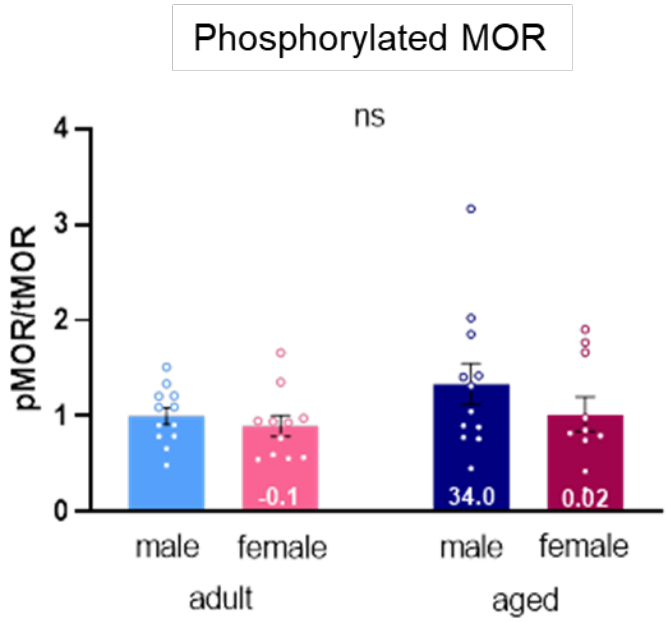
289

290

291

292 **3.3 Advanced age and sex do not impact phosphorylated MOR in the vlPAG**

293 The results above showed significant reductions in MOR binding potential and G-protein activation
294 efficacy. Therefore, we next examined if there was an effect of age and sex on MOR phosphorylation, as
295 increased levels of pMOR would likely contribute to these reductions. Western blots were used to
296 determine the impact of advanced age on MOR phosphorylation at serine-375, a known site of
297 phosphorylation-mediated desensitization (Schulz et al., 2004). There was no significant impact of
298 treatment, so CFA and handled groups were combined. Although increased pMOR/tMOR was observed
299 in aged males compared to their adult counterparts (%Δ 34.0), no significant main effect of age [$F_{(1,41)}$ =
300 2.168, p = 0.1486], sex [$F_{(1,41)}$ = 1.807, p = 0.1863], or age x sex interaction [$F_{(1,41)}$ = 0.4615, p = 0.5008]
301 was observed (Fig. 3).




302

303 **Fig 3.** No significant impact of advanced age or biological sex on the ratio of phosphorylated MOR/ total
304 MOR. ns, not significant. Graph indicates mean ± SEM. Values indicate % change from adult male.

305

306

307 **3.4 Advanced age and sex impact opioid-induced cAMP inhibition in the vlPAG**

308 We next determined if advanced age, biological sex, or persistent pain impacted opioid-induced cAMP
309 inhibition in the vlPAG. Forskolin-induced cAMP release was used as a baseline measurement for each
310 group, while forskolin + DAMGO was used to assess the degree to which cAMP was inhibited by
311 DAMGO. Percent change from baseline was used to compare across treatment groups (Fig. 4A). There
312 was no significant impact of persistent pain on any measures, so CFA and handled groups were
313 combined. Our analyses revealed a significant main effect of age [$F_{(1,30)} = 9.314$, $p = 0.0047$], a significant
314 main effect of sex [$F_{(1,30)} = 10.31$, $p = 0.0031$], and a significant interaction [$F_{(1,30)} = 14.24$, $p = 0.0007$].
315 Post hoc analyses showed a significant difference in cAMP inhibition between adult males and adult
316 females ($p = 0.0002$), adult males and aged males ($p = 0.002$), and adult males and aged females ($p =$
317 $0.004$), indicating that DAMGO elicits greater cAMP inhibition in adult males compared to aged males
318 and females (both adult and aged) (Fig. 4B).

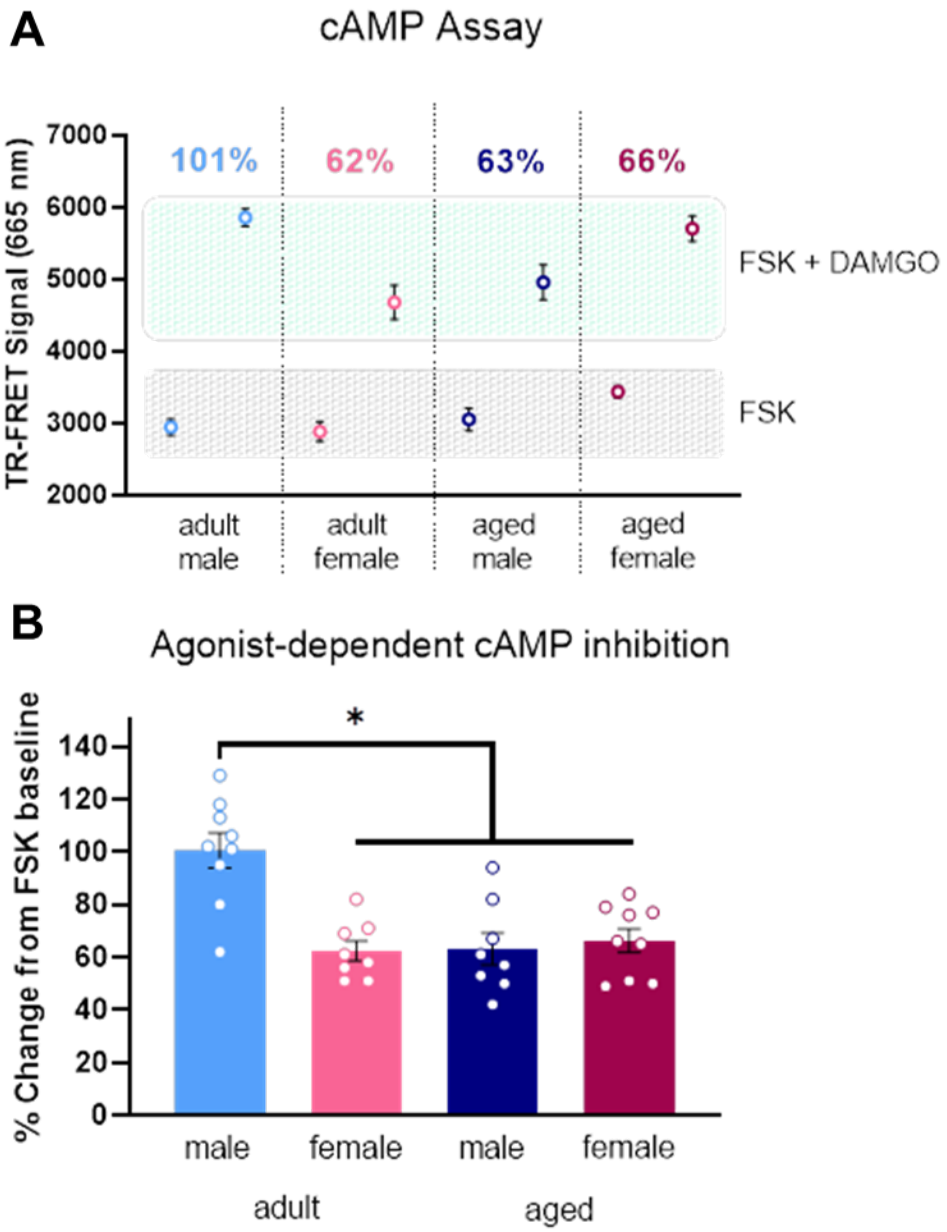



**Fig 4.** TR-FRET immunoassay showing % change from FSK stimulated baseline and DAMGO inhibited cAMP (cAMP levels are inversely proportional to TR-FRET signal). Adult males exhibited the highest level of agonist-dependent % change in cAMP (A). Aged males and females exhibited reduced agonist-dependent cAMP inhibition compared to adult males, indicating attenuated downstream opioid signaling (B). *Significant difference between adult and aged males, or adult males and adult females; $p<0.05$ calculated by Tukey's post hoc test. Graphs indicate mean ± SEM. Values indicate % change from FSK baseline.

328 **3.5 Advanced age and sex impact RGS4 and RGS9-2 expression in the vlPAG**

329 Regulator of G-protein Signaling (RGS) proteins act as GAP accelerators to negatively modulate G-
330 protein signaling. RGS protein family members RGS4 and RGS9-2 are expressed in the vlPAG and have
331 both been shown to regulate opioid signaling by reversing G-protein activation. Therefore, we next used
332 smFISH to determine if RGS4 and RGS9-2 expression in the vlPAG was altered by advanced age and
333 biological sex. In these studies, following CFA administration, a cohort of rats was administered
334 morphine to examine the relationship between morphine $EC_{50}$ and RGS levels. However, no differences
335 were noted between the morphine and naive conditions, so these groups were combined. First, we
336 assessed total vlPAG expression of RGS4 and RGS9-2. These analyses revealed a significant main effect
337 of age on both RGS4 [$F_{(1,37)} = 18.15$, $p = 0.0001$] and RGS9-2 [$F_{(1,41)} = 17.09$, $p < 0.0002$], with aged rats
338 exhibiting increased levels of RGS4 and RGS9-2 mRNA compared to their adult counterparts (Fig. 5A &
339 B). No significant main effect of sex on RGS4 [$F_{(1,37)} = 0.0247$, $p = 0.8763$] or RGS9-2 [$F_{(1,41)} = 0.4855$, $p =$
340 $0.4903$], or significant interactions between age and sex for RGS4 [$F_{(1,37)} = 0.4994$, $p = 0.4842$] or RGS9-2
341 [$F_{(1,41)} = 0.3246$, $p = 0.5723$] were observed (Fig. 5A & B).

342 Following our assessment of total RGS4 and RGS9-2, we next restricted our analyses to RGS4 and RGS9-2
343 mRNA expressed on MOR+ neurons. Similar to what was noted above, a significant main effect of age on
344 expression of RGS4 [$F_{(1,41)} = 26.47$, $p < 0.0001$] and RGS9-2 [$F_{(1,41)} = 21.69$, $p < 0.0001$] was observed, with
345 aged rats exhibiting increased levels of RGS4 and RGS9-2 mRNA in MOR+ neurons compared to their
346 adult counterparts (Fig. 5C & D). No main effect of sex for RGS4 [$F_{(1,41)} = 0.7881$, $p = 0.3799$] or RGS9-2
347 [$F_{(1,41)} = 0.0006$, $p = 0.9799$], or significant interactions between age and sex for RGS4 [$F_{(1,41)} = 0.6583$, $p =$
348 $0.4219$] or RGS9-2 [$F_{(1,41)} = 0.0075$, $p = 0.9314$] were observed (Fig. 5C & D).

349

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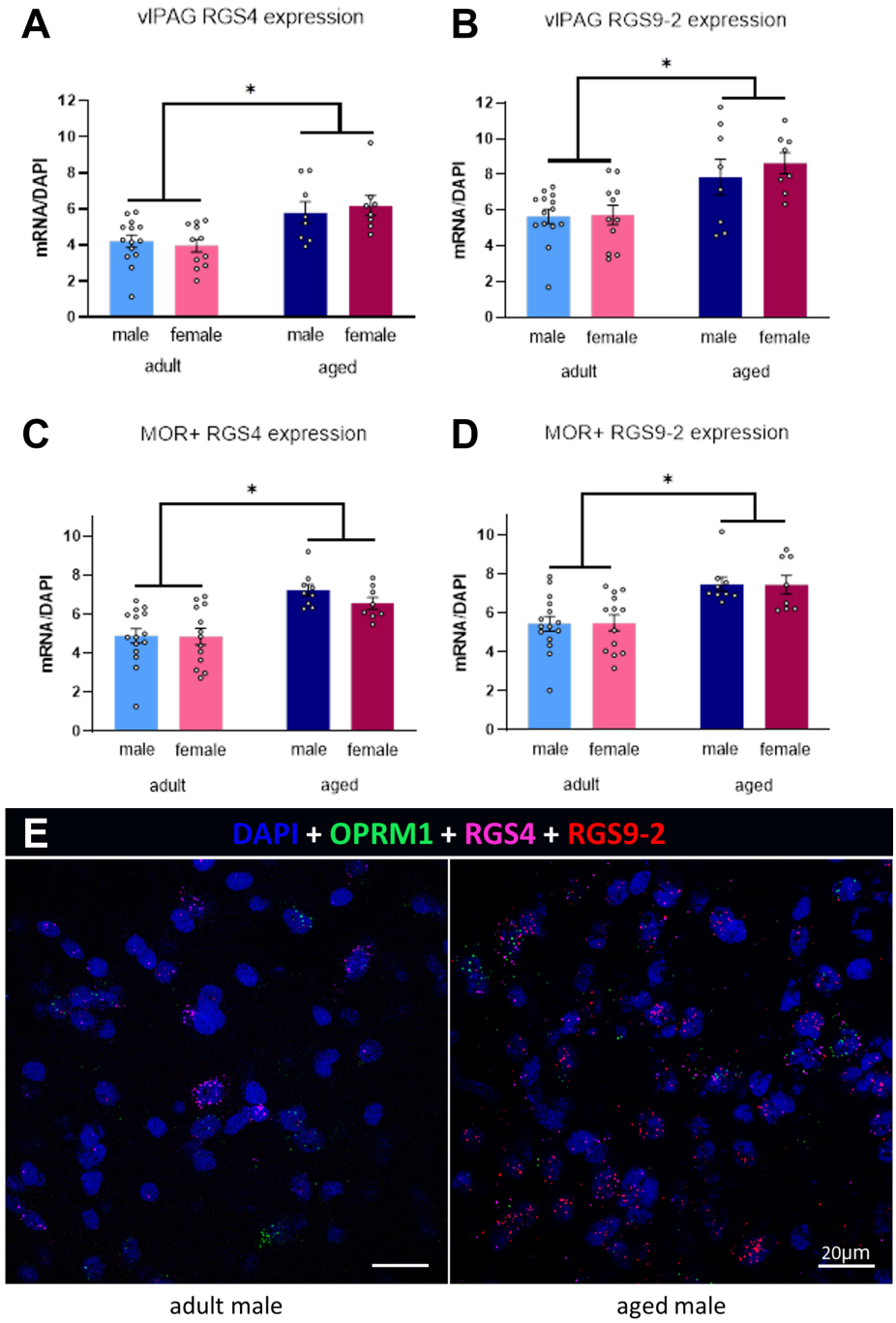
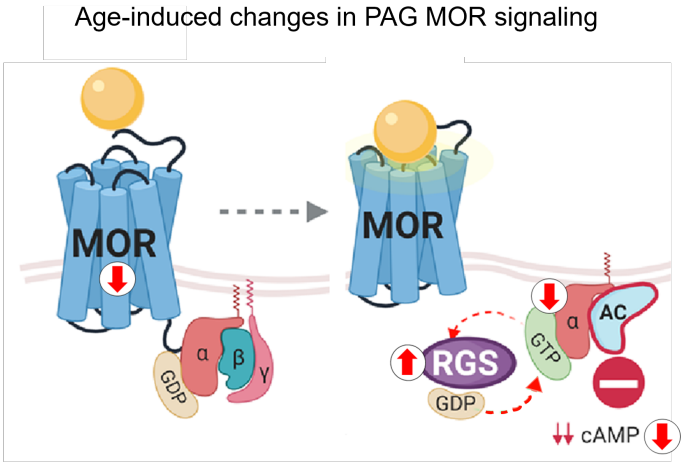



**Fig 5.** mRNA expression of RGS4 (A) and RGS9-2 (B) in the vlPAG was significantly greater in aged rats compared to adults. mRNA expression of RGS4 (C) and RGS9-2 (D) on MOR+ cells in the vlPAG was significantly greater in aged rats compared to adults. (E) Photomicrograph of RGS expression compared an adult male and an aged male PAG section. *Significant difference between adults and aged rats; $p<0.05$ calculated by 2x2 ANOVA. Graphs indicate mean ± SEM.

358 **4 Discussion**

359 The present studies are the first to show that advanced age results in a significant attenuation in MOR
360 signaling within the vlPAG of male and female rats. Specifically, aged males and females (regardless of
361 age) showed decreased MOR binding potential, decreased G-protein activation, and decreased agonist-
362 stimulated cAMP inhibition in comparison to adult males (Fig. 6). These changes, along with the
363 observed increase in RGS4 and RGS9-2 expression, provide a mechanism whereby morphine potency is
364 significantly reduced in aged rats (Fullerton et al., 2021). These changes also support our earlier
365 observed sex difference whereby adult females have a lower opioid analgesic potency than adult males,
366 equivalent to aged males and females.




| | Morphine Potency | Binding Potential | G-Protein Activation | MOR Phosphor. | cAMP Inhibition | RGS mRNA |
|---|---|---|---|---|---|---|
| Adult ♂ | = | = | = | = | = | = |
| Adult ♀ | ↓ | ↓ | ↓ | = | ↓ | = |
| Aged ♂ | ↓ | ↓ | ↓ | = | ↓ | ↑ |
| Aged ♀ | ↓ | ↓ | ↓ | = | ↓ | ↑ |

367

368 **Fig 6.** Model and table of MOR signaling impairments in the vlPAG of the aged and female rat. Aged and
369 female rats exhibit decreased MOR binding potential, decreased G-protein activation, and decreased
370 cAMP inhibition, while aged rats alone demonstrate increased RGS expression. No impact of age or sex
371 was found in phosphorylation of MOR at serine-375.

372

373 **Impact of advanced age and sex on MOR binding potential**

374 In our previous study, we reported that morphine potency to block persistent inflammatory pain is
375 reduced in aged and female rats, likely due to a reduction in DAMGO binding in the vlPAG (Fullerton et
376 al., 2021). Consistent with that finding, we report here that the vlPAG MOR binding potential in aged
377 males and females of either age is significantly reduced in comparison to adult male rats, providing
378 additional support that the decrease in opioid potency observed in these groups is driven, in part, by
379 reduced expression/availability of MOR in the vlPAG. Notably, we did not observe an impact of
380 advanced age or sex on the MOR's affinity for its ligand, as there were no significant effects of age or sex
381 on DAMGO $K_d$ values. However, aged males had reduced MOR availability as evidenced by lower $B_{max}$
382 values compared to their adult male counterparts. This result is consistent with our previous finding that
383 aged males exhibited reduced DAMGO binding in the vlPAG (assessed using autoradiography) (Fullerton
384 et al., 2021), and together, suggest an age-induced downregulation of PAG MOR in males matching a
385 consistently lower level of MOR expression in females of any age. This apparent decrease in MOR
386 expression may explain the reduced analgesic potency of morphine in females and aged males in our
387 earlier work (Fullerton et al., 2021).

388 Here we report no impact of persistent inflammatory pain on vlPAG MOR binding potential, consistent
389 with previous preclinical and clinical studies assessing the impact of chronic pain on MOR binding in the
390 CNS. For example, patients with chronic fibromyalgia pain exhibit no changes in MOR binding in the
391 PAG, despite having significant reductions in several brain regions, including the nucleus accumbens and
392 the amygdala (Harris et al., 2007). Similarly, no change in PAG MOR binding was noted as a result of
393 peripherally localized chronic pain generated from proximal nerve injury (Maarrawi et al., 2007).
394 Interestingly, centrally localized neuropathic pain (generated from supraspinal injury) was associated
395 with a significant reduction of PAG MOR binding, suggesting that the origin of chronic pain within the
396 CNS is critical in promoting alterations in neural MOR signaling (Maarrawi et al., 2007). Preclinical

397 studies in rodents also suggest that changes in MOR induced by persistent pain are CNS site-specific. For
398 example, persistent inflammatory pain induced by intraplantar CFA increased MOR binding in the dorsal
399 root ganglion at 24 and 96 hours post-CFA, (Mousa et al., 2001; Zollner et al., 2003), with no change in
400 MOR noted for the hypothalamus or spinal cord (Shaqura et al., 2004). Similarly, no change in PAG MOR
401 availability or expression was observed following sciatic nerve injury, although reductions were noted
402 for the insula, caudate putamen, and motor cortex (Thompson et al., 2018). Taken together, these
403 results suggest that chronic pain alters MOR signaling in a CNS region-specific manner that is dependent
404 on the site and modality of the pain.

405 **Impact of advanced age and sex on G-protein activation**

406 Agonist binding at MOR activates Gαi/o -proteins and downstream signaling cascades which are critical
407 for opioid-mediated hyperpolarization (Laugwitz et al., 1993; Connor and Christie, 1999; Koehl et al.,
408 2018). In the present study, we found that adult males exhibit greater G-protein activation efficiency
409 compared to aged males and adult and aged females, suggesting that the decreases in opioid potency
410 observed in the aged males and the females are driven, in part, by attenuated G-protein-MOR coupling
411 in the vlPAG. A significant impact of biological sex on G-protein potency ($EC_{50}$) was also observed, with
412 females and aged males showing a reduction in the potency of opioid-dependent G-protein activation,
413 as evidenced by increased $EC_{50}$ values. This reduction in vlPAG G-protein activation likely contributes to
414 previous studies reporting that females are less sensitive to the analgesic effects of opioids (Kest et al.,
415 2000; Loyd et al., 2008; Wang et al., 2014; Fullerton et al., 2018). Although the mechanism by which this
416 change in potency occurs is not known, the nature of the GTPγS assay is suggestive. The assay uses
417 membrane preparations without soluble signaling regulators like kinases present. Similarly, the GTPγS
418 molecule itself is not hydrolyzable, and is thus not subject to regulation by GAPs like the RGS proteins.
419 Considering these factors, the observed decrease in G protein activation potency may be due to the
420 observed decrease in opioid receptor expression/availability in the binding studies above.

421

422 Interestingly, this was the only assay where an impact of persistent inflammatory pain was observed.
423 Specifically, CFA treated rats had reduced $EC_{50}$ values, regardless of age or sex. Reductions in Gα subunit
424 expression have also been reported within both the rostral ventromedial medulla and spinal cord dorsal
425 horn following intraplantar CFA in adult male rats (Wattiez et al., 2017). The mechanism whereby
426 persistent inflammatory pain would impact G-protein activation, and not the other pharmacodynamics
427 of MOR, is not known. Also unknown are the mechanisms by which advanced age and sex alter G-
428 protein-MOR signaling. Previous studies have reported that advanced age results in a global
429 downregulation of Gαi/o, most notably in the prefrontal cortex (Young et al., 1991; Alemany et al., 2007;
430 de Oliveira et al., 2019), that is not associated with overall cell loss. Indeed, the Gαi/o subunit in
431 particular appears to be highly susceptible to the aging process, with estimates of reduced expression as
432 high as 65% in the frontal cortex, hippocampus, substantia nigra, and striatum (de Oliveira et al., 2019).
433 Although age-induced changes in Gαi/o expression were not assessed specifically in PAG, a widespread
434 reduction in Gαi/o as a function of advanced age would likely impact the PAG, thereby limiting MOR-G-
435 protein coupling and reducing both G-protein activation efficiency and opioid potency in the aged rat.
436 Alternatively, an uncoupling between MOR and Gαi/o and/or a switch in Gα subunit from Gαi/o to Gαs
437 cannot be ruled out (Gintzler and Chakrabarti, 2000, 2004, 2006). Indeed, a shift from Gαi/o to Gαs
438 would similarly increase adenylate cyclase activity, resulting in reduced hyperpolarization and decreased
439 morphine potency. Clearly, additional studies assessing the impact of advanced age on the expression of
440 Gα isoforms are warranted (Lamberts et al., 2011).
441 The present studies used the high-affinity MOR agonist DAMGO for both the radioligand binding and
442 GTPyS assay. As previous studies have reported that the pharmacodynamics of MOR may be agonist-
443 dependent, this possibility cannot be ruled out and future studies should consider incorporating at least
444 two agonists in all assays. Alternatively, a contribution by OPRM1 splice variants induced as a function of

445 age may also be present (Pan et al., 2005; Alvarez et al., 2002; Narayan et al., 2021). Indeed, specific
446 alternative MOR isoforms are known to promote ligand-dependent regulation of opioid signaling
447 (Pasternak et al., 2004; Oldfield et al., 2008). MOR-1B, a family of MOR isoforms expressed in the brain,
448 are indistinguishable from MOR-1 at the extracellular surface and the agonist binding pocket but elicit
449 alternative signaling properties that impact opioid-induced cell hyperpolarization, and dysregulation of
450 these MOR splice variants has been implicated in advanced age (Latorre and Harries, 2017; Li et al.,
451 2017; Wang et al., 2018). There are also reports of sex differences in the expression of MOR splice
452 variants in the PAG, with male rodents expressing higher levels of MOR-1B5, and females expressing
453 higher levels of MOR-1B1 and MOR-1B2 (Liu et al., 2018). The unique conformation of MOR isoforms
454 and subsequent alterations in receptor phosphorylation would directly influence the G-proteins to
455 which MORs couple, potentially resulting in biased agonism (Verzillo et al., 2014; Abrimian et al., 2021).

456 **Impact of advanced age and sex on MOR Phosphorylation**

457 The results of the radioligand binding and GTPγS assays suggested that aged and female rats exhibit
458 decreased receptor expression and activation potency. It has been previously shown that MOR can be
459 desensitized by phosphorylation in its basal state, thereby limiting agonist activation (Zhang et al., 1996;
460 Yu et al., 1997; Groer et al., 2011). Thus, we tested the hypothesis that the observed reductions in G-
461 protein activation efficiency were due to age- and sex-induced changes in basal MOR phosphorylation.
462 Although aged rats expressed higher levels of MOR phosphorylation at serine-375 compared to the
463 adults, this result was not significant, suggesting that the reductions in activation potency observed in
464 the vlPAG of aged and female rats is likely driven by an alternative mechanism.

465 MOR desensitization is mediated by G protein-coupled receptor kinase (GRK)-dependent
466 phosphorylation of the receptor, a mechanism by which β-arrestins bind to MOR, resulting in
467 endocytosis (J. Zhang et al., 1998; Schulz et al., 2004; Dang et al., 2009). Although our results suggest no

468 impact of advanced age or sex on GRK-mediated MOR phosphorylation, MOR signaling is also
469 desensitized via phosphorylation through the extracellular signal-regulated kinase 1 and 2 (ERK1/2)
470 pathway (Dang et al., 2009). ERK1/2 phosphorylation stimulates the activity of Gα-interacting protein
471 (GAIP), an RGS protein that acts as a GTPase activator to reduce opioid signaling at the level of G-protein
472 activation (Ogier-Denis et al., 2000). Indeed, pharmacological inhibition of ERK1/2 phosphorylation in a
473 rat model leads to improved morphine analgesia (Popiolek-Barczyk et al., 2014; Melkes et al., 2020).
474 Thus, the age- and sex-induced changes in morphine potency may be driven in part by age-induced
475 hyper-phosphorylation of ERK1/2 and result in the downregulation of G-protein signaling.

476 **Impact of advanced age and sex on cAMP inhibition**

477 Agonist binding at MOR elicits a conformational change in the receptor that allows the G-protein α and
478 βγ subunits to interact with downstream effectors. Notably, the Gα subunit binds to adenylate cyclase
479 (AC) and inhibits the conversion of adenosine triphosphate (ATP) to cAMP, thus limiting the activation of
480 cAMP-dependent protein kinase (PKA) and ultimately inducing higher levels of hyperpolarization
481 (Christie, 2008; Santhappan et al., 2015). In the present study, aged males and adult and aged females
482 exhibited significantly lower levels of DAMGO-induced cAMP inhibition compared to adult males,
483 suggesting an attenuated activity of the α subunit at the level of AC, or a weakened relationship
484 between AC and cAMP. This effect could easily be due to the observed decreases in MOR expression and
485 activation potency observed above. This attenuated cAMP inhibition in the aged and female rats likely
486 contributes to the observed reduction in opioid potency and may also be due, in part, to decreased
487 basal cAMP levels in the aged brain. Reductions in cAMP have been reported in several rodent brain
488 regions including the cortex, thalamus, hypothalamus, and midbrain (Puri and Volicer, 1981; Titus et al.,
489 2013; Kelly, 2018). Additional evidence suggests that aged rodents elicit widespread reductions in ATP
490 (Błaszczyk, 2020), which is required for cAMP synthesis. Not surprisingly, previous studies assessing the

491 impact of advanced age on cAMP expression and signaling were conducted exclusively in male rodents,
492 so it is not known whether advanced age impacts neural cAMP levels in a sex-specific manner.

493 Morphine binding to MOR results in the activation of Gai/o that primarily inhibits the activity of AC,
494 thereby reducing the ability to generate cAMP; in parallel, the βγ subunits activate G-protein inwardly
495 rectifying potassium (GIRK) channels to induce hyperpolarization and decrease excitability (Law et al.,
496 2000; Nobles et al., 2005; Yudin and Rohacs, 2018). Advanced age is concomitant with increased levels
497 of neuroinflammation and greater production of reactive oxygen species (ROS), both of which have been
498 implicated in age-induced potassium channel dysfunction (Sparkman and Johnson, 2008; Sesti, 2016;
499 Lieberman et al., 2020). Consistent with this, aged Sprague Dawley rats (26-30 mos) exhibit reduced
500 potassium currents in hippocampal neurons (Alshuaib et al., 2001). Further experimentation assessing
501 the impact of advanced age and sex on GIRK channel-mediated hyperpolarization, in addition to cAMP-
502 mediated hyperpolarization, will provide a more detailed description of the impact of advanced age and
503 sex on downstream opioid signaling in the PAG.

504 **Impact of advanced age and sex on RGS protein levels**

505 G-protein activation by MOR results in the exchange of GDP for GTP (Senese et al., 2020). GTP-bound Gα
506 proteins are inactivated by hydrolysis of the GTP back to GDP, catalyzed by the intrinsic GTPase activity
507 of Gα proteins. This inactivation mechanism is modulated by the activity of Regulator of G-protein
508 signaling (RGS) proteins. RGS proteins reduce the magnitude of GPCR signaling via enhancement of
509 GTPase activity of the α subunit of the G-protein complex. By promoting the hydrolysis of the alpha-
510 bound GTP during the active state, RGS proteins hasten the return of the α subunit to the GDP-bound
511 inactive state (Roman and Traynor, 2011). RGS proteins play a critical role in negatively modulating
512 opioid signaling, as morphine analgesia is increased in male mice lacking RGS9-2 (Garzón et al., 2001;
513 Zachariou et al., 2003) and overexpression of RGS4 attenuates MOR signaling in reconstituted MORs in

514 vitro (Ippolito et al., 2002). In the present study, we observed increased expression of RGS4 and RGS9-2
515 in the vlPAG of aged rats compared to adults, suggesting greater GTPase activity and thus reduced G-
516 protein signaling in the aged. Similar results were observed when the analysis was limited to MOR+ cells,
517 indicating that opioid-induced G-protein signaling is subjected to greater negative regulation in the
518 vlPAG of the aged rat. These results are consistent with Kim et al. (2005) who reported higher RGS9-2
519 protein levels in the PAG of 1-year old male rats compared to 3-week-old rats (Kim et al., 2005). In
520 humans, advanced age is associated with increased RGS4 expression in the prefrontal cortex (Rivero et
521 al., 2010), suggesting that the age-induced increase in RGS4 in our experiment may not be specific to the
522 vlPAG. These results provide a mechanism by which advanced age could decrease analgesic potency and
523 downstream cAMP signaling, although they do not provide a mechanism by which adult females
524 demonstrate lower potency and signaling than adult males.

525 **Summary and Conclusions**

526 The present studies are the first to show that sex and advanced age leads to an attenuation in vlPAG
527 opioid signaling compared to adult male rats. Taken together with our previous findings, these results
528 suggest that age- and sex-induced reductions in vlPAG MOR expression and binding, combined with
529 attenuated downstream MOR signaling, contribute to the diminished opioid potency reported in aged
530 and female rats (Fullerton et al., 2021). The results of our analyses demonstrate that aged and female
531 rats exhibit reductions in MOR expression/availability, G-protein activation, and cAMP inhibition, and
532 increased G-protein regulation by RGS proteins, each of which provides potential therapeutic targets for
533 improved pain management in the elderly.

534

535 The nature of the results observed also suggest potential mechanisms of action for sex and age to
536 impact analgesic potency. First, the normal ligand affinity (Kd) and lack of receptor phosphorylation

537 suggests that unit receptor performance is normal. When combined with the observed decrease in
538 expression/availability (Bmax), this suggests that one major mechanism is a decrease in receptor
539 expression instead of a change in unit receptor performance. When considering the G protein coupling
540 analysis, since soluble regulators (like RGS proteins) are absent in this assay and the $^{35}$S-GTPγS is non-
541 hydrolyzable, the decrease in G protein coupling potency is likely due to decreased receptor availability.
542 Downstream of the G proteins, we also observed a decrease in cAMP inhibition. This downstream effect
543 could be due to the observed increase in RGS expression, decreasing G protein activity, leading to less
544 cAMP inhibition. However, RGS expression was not increased in adult females. Overall then our results
545 suggest two major complementary mechanisms; 1) decreased receptor expression/availability in adult
546 females and aged males and females, and 2) increased RGS expression in aged males and females.
547 Further investigation could also uncover additional complementary sex- and age-related mechanisms
548 that help explain why opioids show lower analgesic potency in females and the aged.

549

550

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556 the data.

557

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849 **Figure Legends**

850 **Fig. 1.** Saturation binding curve of bound [3H]DAMGO (A). No significant effect of age or sex was
851 observed in MOR affinity ($K_d$) (B). MOR availability ($B_{max}$) was significantly lower in aged males compared
852 to adult males (C). Adult females, aged males, and aged females exhibited reduced MOR binding
853 potential ($B_{max}/K_d$) compared to adult males, indicating attenuated agonist interaction at the receptor
854 level (D). ns, not significant. *Significant difference between adult and aged males, or adult males and
855 adult females; $p<0.05$ calculated by Tukey's post hoc test. Graphs indicate mean ± SEM. Values indicate
856 % change from adult male.

857 **Fig 2.** [$^{35}$S]GTPγS binding curve of agonist-stimulated GTPγS binding (A). No significant effect of age or
858 sex was observed in $E_{max}$ (B). Aged males, adult females, and aged females exhibited increased $EC_{50}$
859 values compared to adult males, indicating that adult males have the greatest potency (C). Aged males,
860 adult females, and aged females exhibited reduced G-protein activation efficiency ($E_{max}/EC_{50}$) compared
861 to adult males, indicating attenuated G-protein signaling (D). ns, not significant. *Significant difference
862 between adult and aged males, or adult males and adult females; $p<0.05$ calculated by Tukey's post hoc
863 test. Graphs indicate mean ± SEM. Values indicate % change from adult male.

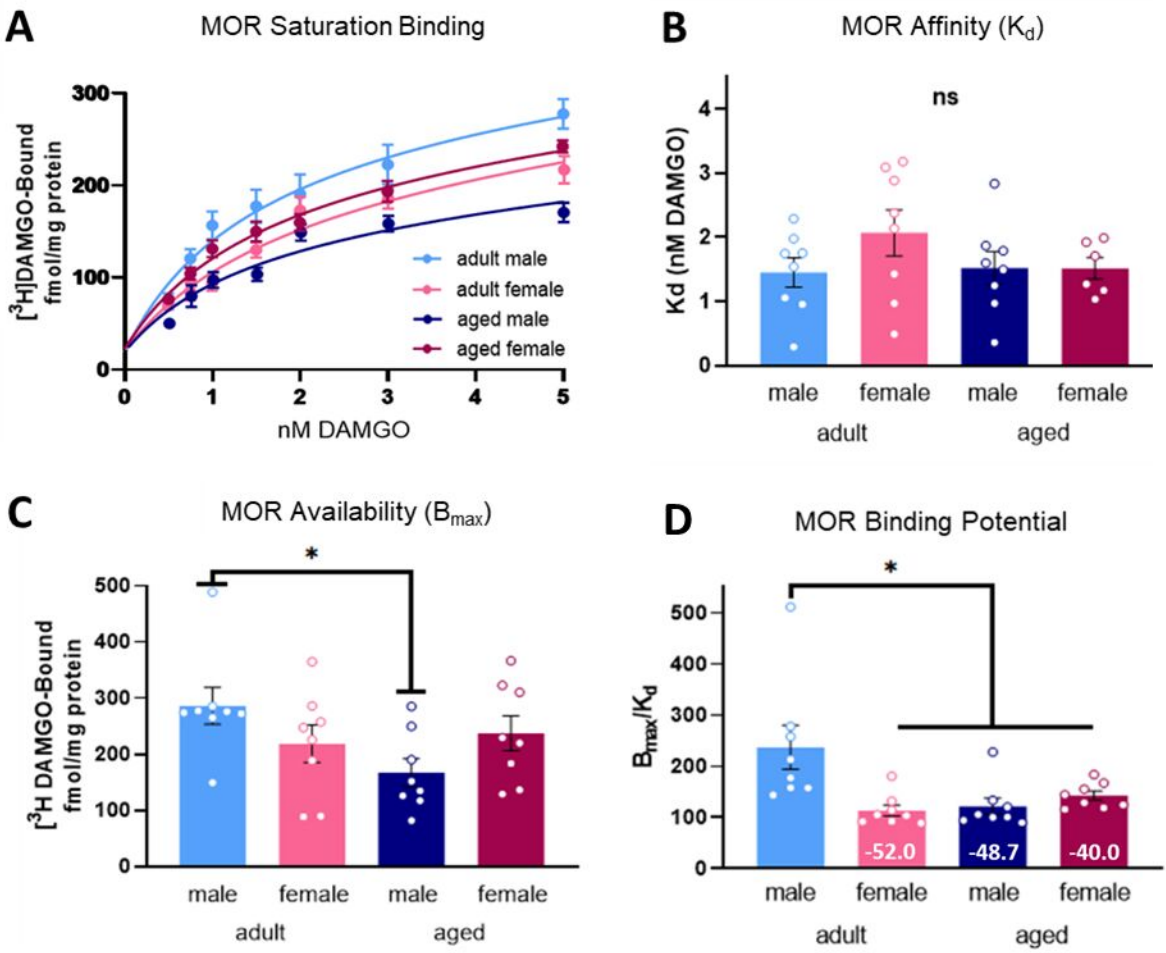
864 **Fig 3.** No significant impact of advanced age or biological sex on the ratio of phosphorylated MOR/ total
865 MOR. ns, not significant. Graph indicates mean ± SEM. Values indicate % change from adult male.

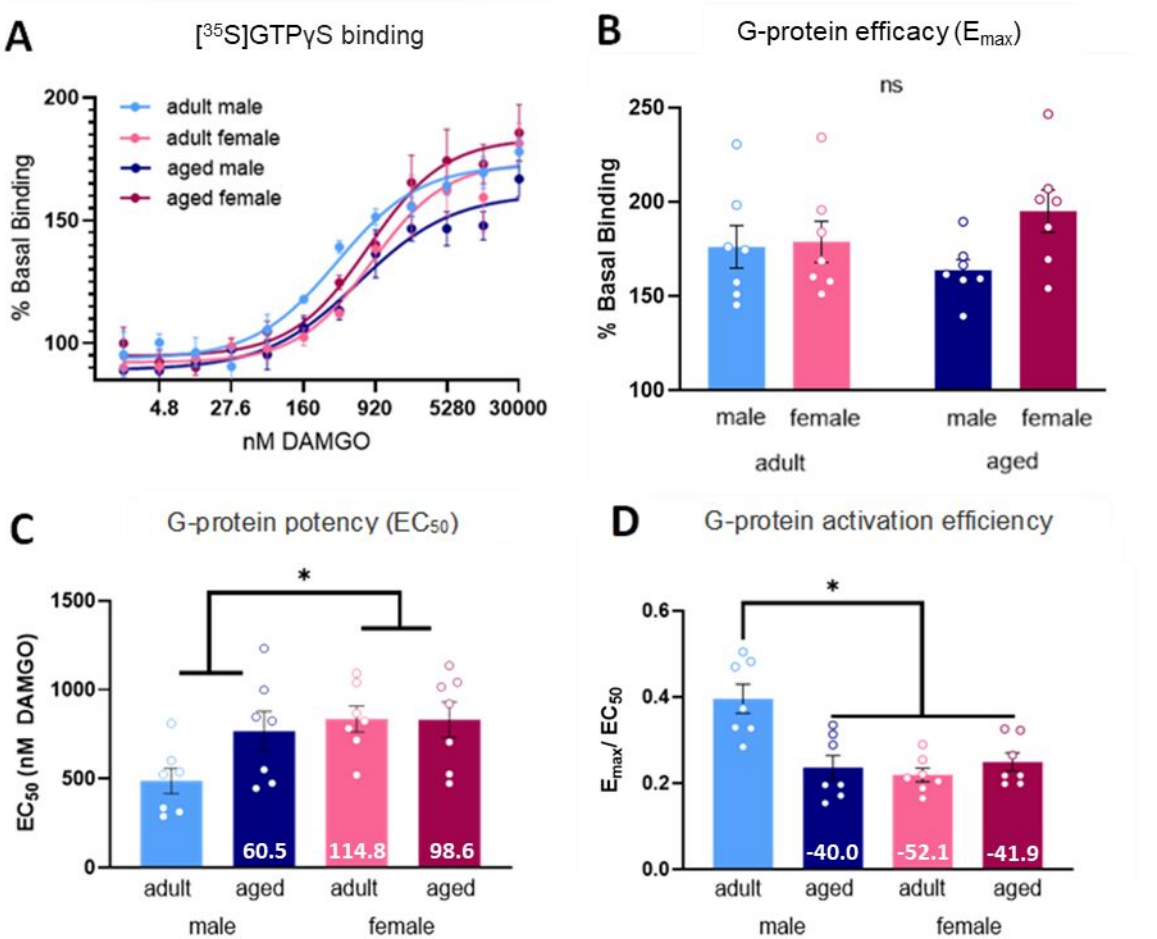
866 **Fig 4.** TR-FRET immunoassay showing % change from FSK stimulated baseline and DAMGO inhibited
867 cAMP (cAMP levels are inversely proportional to TR-FRET signal). Adult males exhibited the highest level
868 of agonist-dependent % change in cAMP (A). Aged males and females exhibited reduced agonist-
869 dependent cAMP inhibition compared to adult males, indicating attenuated downstream opioid
870 signaling (B). *Significant difference between adult and aged males, or adult males and adult females;
871 $p<0.05$ calculated by Tukey's post hoc test. Graphs indicate mean ± SEM. Values indicate % change from
872 FSK baseline.

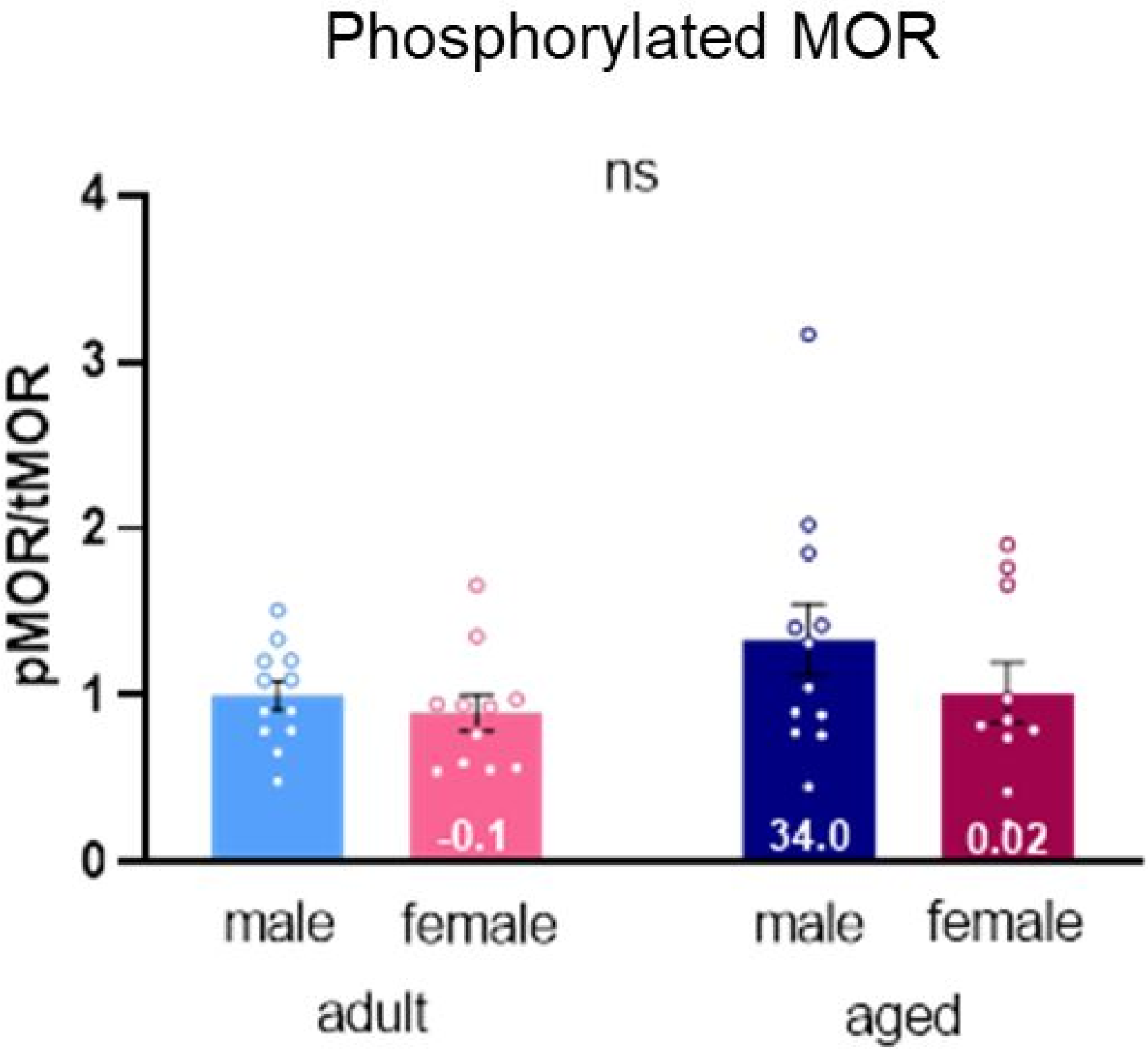
873 **Fig 5.** mRNA expression of RGS4 (A) and RGS9-2 (B) in the vlPAG was significantly greater in aged rats
874 compared to adults. mRNA expression of RGS4 (C) and RGS9-2 (D) on MOR+ cells in the vlPAG was
875 significantly greater in aged rats compared to adults. (E) Photomicrograph of RGS expression compared
876 an adult male and an aged male PAG section. *Significant difference between adults and aged rats;
877 $p<0.05$ calculated by 2x2 ANOVA. Graphs indicate mean ± SEM.

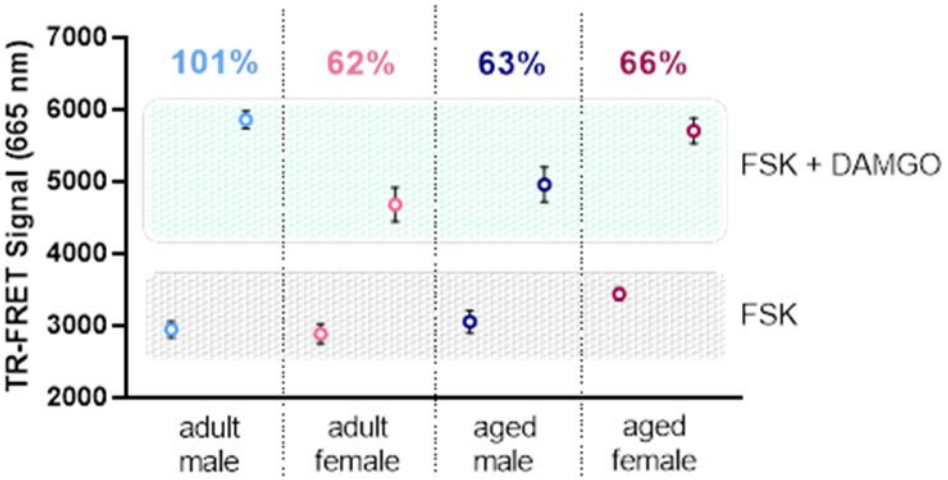
878 **Fig 6.** Model and table of MOR signaling impairments in the vlPAG of the aged and female rat. Aged and
879 female rats exhibit decreased MOR binding potential, decreased G-protein activation, and decreased
880 cAMP inhibition, while aged rats alone demonstrate increased RGS expression. No impact of age or sex
881 was found in phosphorylation of MOR at serine-375

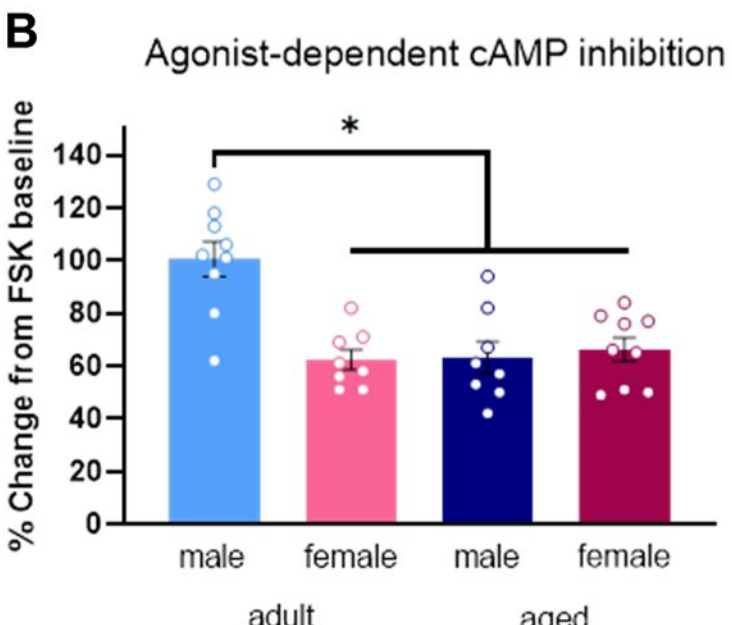
882

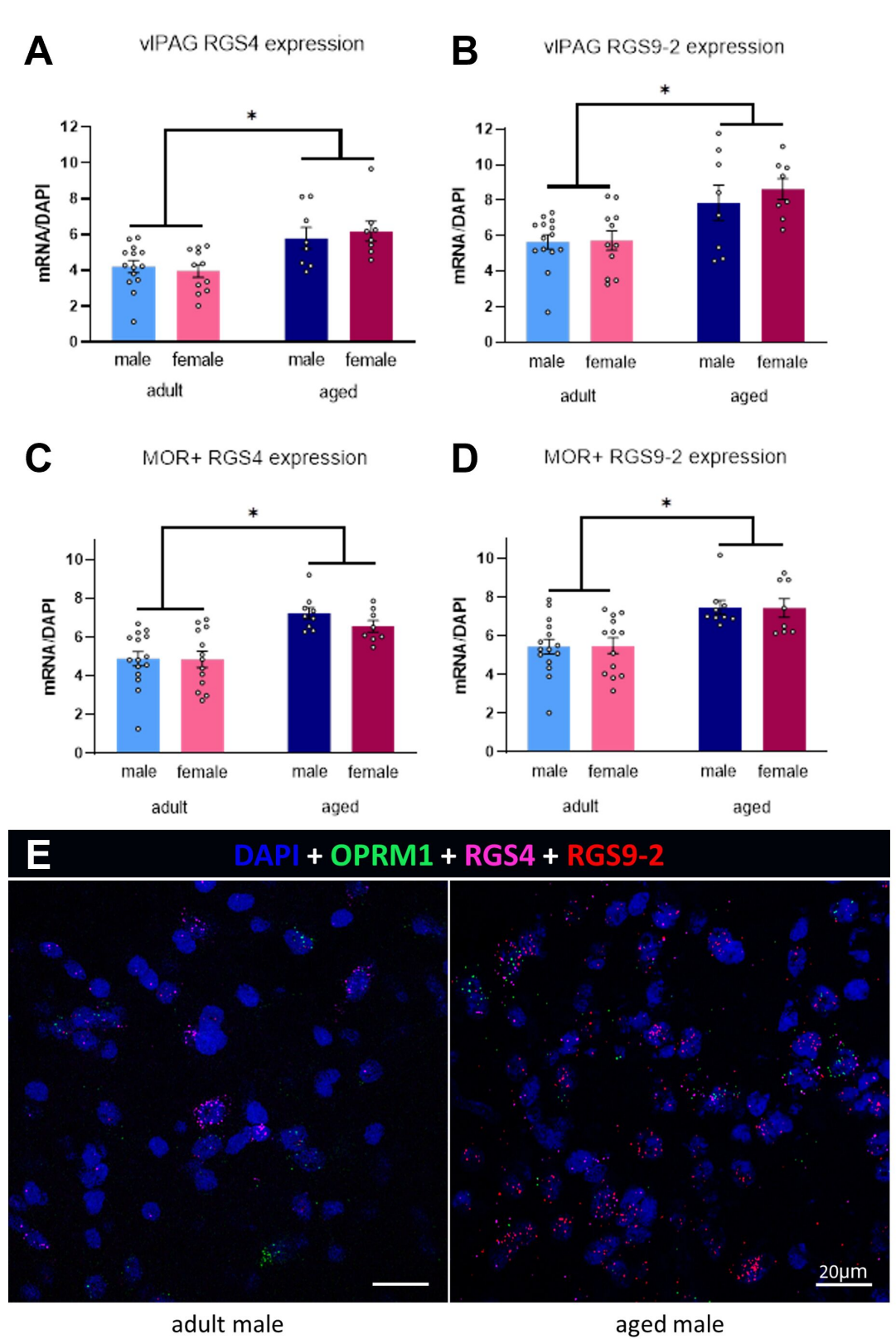

A
MOR Saturation Binding
[3H]DAMGO-Bound fmol/mg protein
300
200
100
0
0
1
2
3
4
5
nM DAMGO
adult male
adult female
aged male
aged female
B
MOR Affinity (Kd)
Kd (nM DAMGO)
ns
4
3
2
1
0
male
female
male
female
adult
aged
C
MOR Availability (Bmax)
[3H DAMGO-Bound fmol/mg protein
*
500
400
300
200
100
0
male
female
male
female
adult
aged
D
MOR Binding Potential
Bmax/Kd
*
500
400
300
200
100
0
-52.0
-48.7
-40.0
male
female
male
female
adult
aged


A
[35S]GTPγS binding
adult male
adult female
aged male
aged female
% Basal Binding
200
150
100
4.8
27.6
160
920
5280
30000
nM DAMGO
B
G-protein efficacy (Emax)
ns
% Basal Binding
250
200
150
100
male
female
male
female
adult
aged
C
G-protein potency (EC50)
*
EC50 (nM DAMGO)
1500
1000
500
0
60.5
114.8
98.6
adult
aged
adult
aged
male
female
D
G-protein activation efficiency
*
Emax/ EC50
0.6
0.4
0.2
0.0
-40.0
-52.1
-41.9
adult
aged
adult
aged
male
female

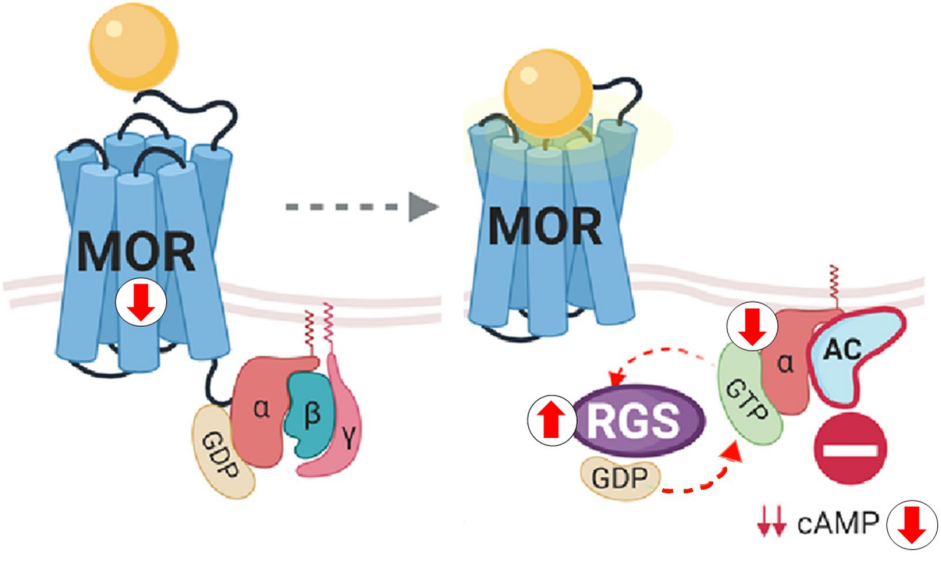

Phosphorylated MOR
ns
pMOR/tMOR
4
3
2
1
0
-0.1
34.0
0.02
male
female
male
female
adult
aged


A
cAMP Assay
TR-FRET Signal (665 nm)
7000
6000
5000
4000
3000
2000
101%
62%
63%
66%
FSK + DAMGO
FSK
adult male
adult female
aged male
aged female


B
Agonist-dependent cAMP inhibition
% Change from FSK baseline
140
120
100
80
60
40
20
0
*
male
female
male
female
adult
aged


A
vlPAG RGS4 expression
mRNA/DAPI
0
2
4
6
8
10
12
*
male
female
male
female
adult
aged
B
vlPAG RGS9-2 expression
mRNA/DAPI
0
2
4
6
8
10
12
*
male
female
male
female
adult
aged
C
MOR+ RGS4 expression
mRNA/DAPI
0
2
4
6
8
10
*
male
female
male
female
adult
aged
D
MOR+ RGS9-2 expression
mRNA/DAPI
0
2
4
6
8
10
*
male
female
male
female
adult
aged
E
DAPI + OPRM1 + RGS4 + RGS9-2
20μm
adult male
aged male

# Age-induced changes in PAG MOR signaling




| | Morphine Potency | Binding Potential | G-Protein Activation | MOR Phosphor. | cAMP Inhibition | RGS mRNA |
|---|---|---|---|---|---|---|
| Adult ♂ | = | = | = | = | = | = |
| Adult ♀ | ↓ | ↓ | ↓ | = | ↓ | = |
| Aged ♂ | ↓ | ↓ | ↓ | = | ↓ | ↑ |
| Aged ♀ | ↓ | ↓ | ↓ | = | ↓ | ↑ |